

Temporal synergism by microencapsulation of piperonyl butoxide and α -cypermethrin overcomes insecticide resistance in crop pests

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Abstract: A microencapsulated formulation that gives a burst release of piperonyl butoxide (PBO) several hours before a burst release of a conventional pyrethroid can effectively overcome metabolic resistance in *Bemisia tabaci* Gennadius, *Helicoverpa armigera* (Hübner), *Aphis gossypii* Glover and *Myzus persicae* Sulzer. This increase in efficacy against resistant pests was reflected in a field trial against *B. tabaci* on cotton, eliminating the need for two treatments. The ratio between the active insecticide and the synergist was found to be crucial in reducing resistance factors.

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Keywords: temporal synergism; resistance; esterase; piperonyl butoxide; microencapsulation

1 INTRODUCTION

Insecticide resistance is a widespread phenomenon whereby insects that are subjected to intense chemical treatment may evolve mechanisms to protect themselves, and hence are able to survive and pass their resistance genes on to their offspring.¹ Resistance can then spread quickly through insect populations by virtue of short generation times and high rates of fecundity.² Resistance can occur either by mutations that change the target-site proteins or by changes in the insect's ability to detoxify insecticides using enzyme systems such as esterases, microsomal oxidases and glutathione-S-transferases (GSTs).³ The last 10 years have seen extensive progress in understanding the molecular basis, genetics and genomics of resistance.^{4,5} Many mutations that confer insensitive target sites have now been identified, and the wide range of insect microsomal oxidases, GSTs and esterases^{6–8} capable of detoxifying insecticides have been elucidated. Such metabolic resistance is of great concern, as it is often capable of conferring resistance across a range of insecticides,⁹ whereas target-site resistance more commonly only confers cross-resistance to other insecticides within the same chemical class, e.g. knockdown resistance (kdr),¹⁰ or even to specific insecticides, e.g. modified acetylcholinesterase (MACE).¹¹

In the peach-potato aphid, *Myzus persicae* Sulzer, at least three resistance mechanisms have evolved,¹² including that of enhanced esterase activity.¹³ This

mechanism is also found in the cotton aphid, *Aphis gossypii* Glover,^{14,15} the tobacco whitefly *Bemisia tabaci* Gennadius¹⁶ and the cotton bollworm *Helicoverpa armigera* (Hübner).¹⁷ Indeed, for many of the widely used pyrethroid insecticides, resistance in field populations of several crop pest species may be primarily due to esterase isoenzymes that can hydrolyse and/or sequester the toxins.

It has long been reported that piperonyl butoxide (PBO) can 'synergise' the action of insecticides by inhibiting microsomal oxidases.^{18,19} It has more recently been established that PBO can also inhibit non-specific esterases,¹⁷ and, using laboratory bioassays, PBO was shown to potentiate pyrethroid activity against resistant laboratory strains of *B. tabaci*, *H. armigera*, *A. gossypii* and *M. persicae* where the resistance was due to metabolic factors.²⁰ Furthermore, in *H. armigera* and *B. tabaci*, an application of PBO at least 5 h before application of the pyrethroids α -cypermethrin and lambda-cyhalothrin resulted in effective control of highly resistant insects.²¹

Thus it would be anticipated that, in the field, pretreatment with PBO followed several hours later by a pyrethroid would also allow control of other insect pests that had evolved resistance by enhanced esterase(s) and/or microsomal oxidases. However, the need for two separate sprays of crops is not always appropriate or even financially viable; one solution has been to use the concept of insecticide microencapsulation to delay insecticide release until the factors causing resistance have been effectively

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inhibited by immediately available PBO.²² For the present work, a formulation of PBO and pyrethroid that allows a burst release of the PBO before a second burst release of the pyrethroid some hours later has been developed on the basis of beta-cyclodextrin microencapsulation.²³ The effects of such microencapsulated formulations containing PBO and α -cypermethrin against pyrethroid-resistant *B. tabaci*, *H. armigera*, *A. gossypii* and *M. persicae* are reported here.

Following the testing of the initial microencapsulated PBO and α -cypermethrin, the formulation was optimised. It appeared that the ratio of PBO to α -cypermethrin had a direct effect on mortality – the higher the ratio of PBO, the higher was the mortality. The effects on synergism of altering the amount of PBO has previously been noted – increasing the dose of synergist enhanced the synergistic ratio when synergist and insecticide were coapplied.²⁴

2 MATERIALS AND METHODS

2.1 Insects

2.1.1 *Bemisia tabaci*

All strains were maintained on cotton plants (*Gossypium hirsutum* L. var. Deltapine 16 and Sicot 189) at 26 ± 2 °C with a 16:8 h light:dark photoperiod. Standard susceptible strains were SUD-S collected from the Sudan in 1978 and DARWIN collected from the Northern Territory, Australia, in 2003; B-type resistant strains were PIRGOS collected from Cyprus in 2003 and EMERALD collected from the cotton in Queensland, Australia, in 2003; CHLORAKA is a Q-type resistant strain collected from Cyprus in 2003. To ensure good insect quality, only adults less than 10 days old were used.

2.1.2 *Helicoverpa armigera*

Strain HAC with elevated esterase was established from eggs collected from cotton crops in New South Wales and Queensland, Australia, in 2002/2003. The strain has been selected by exposure to insecticides in the field, including pyrethroids. A pyrethroid-susceptible strain collected from cotton at Narrabri, New South Wales, in 1982 was used for comparison.

The strains were reared on a modified Shorey Hale artificial diet at 25 ± 2 °C and 80% RH in natural light.

2.1.3 *Myzus persicae*

Clones were reared on three-week-old Chinese cabbage plants (*Brassica pekinensis* (Lour) Rup. var. Wong Bok) maintained at 18 ± 2 °C with a 16:8 h light:dark photoperiod. The standard susceptible clone US1L was collected in Bottisham, Cambridge, in 1974. The resistant clone 794JZ is a highly resistant (R_3) variant obtained from a glasshouse at Evesham in 1982.

2.1.4 *Aphis gossypii*

Clones were reared on three-week-old pumpkin plants (*Cucurbita pepo* L. var. Mammoth Gourd) maintained at 18 ± 2 °C with a 16:8 h light:dark photoperiod. Clone 171B was isolated from a population collected from the UK in 1981 and maintained as a reference susceptible. The resistant clone 1196 possessing highly elevated esterase was collected from cotton in Greece in 2003.

2.2 Insecticides

Two commercial α -cypermethrin 100 g L⁻¹ EC formulations, Fastac 10EC (BASF) and Dominex 10EC (Crop Care), were used. The PBO formulations used were PBO 30 (microemulsified in water; Endura SpA) for all bioassays except the DARWIN and EMERALD strains of *B. tabaci* where PBO 80 EC (Endura SpA) was applied.

The microencapsulated formulations were developed by Giovanna Delogu at CNR Institute of Biomolecular Chemistry, Sardinia, and by Valerio Borzatta, Endura SpA. All were suspended in distilled water containing 0.1 g L⁻¹ of the non-ionic wetting agent Agral® (Zeneca Agrochemicals) immediately prior to bioassays. The formulation allowed release of the PBO, followed by release of the α -cypermethrin 5 hours later. Contents of each microencapsulated formulation are shown in Table 1.

2.3 Leaf dip bioassays

Leaf dip bioassays were based on those previously published.²⁵ Leaf discs (3 cm) cut from cotton leaves were dipped into serial dilutions (1.0–0.0001 g AI L⁻¹) of each formulation and left to dry. The discs were then placed onto agar (1%) in petri dishes (37 mm diameter, 15 mm high), and either ten second-instar *H. armigera* larvae, ten adult aphids or 20–40 adult whiteflies (anaesthetised with carbon dioxide) were placed on the discs. Each unit was sealed with a close-fitting ventilated lid, and mortalities were assessed after 48 h at standard rearing conditions. For the PBO pretreatment, the insects were placed on leaves treated with 0.5 g L⁻¹ PBO and then transferred to discs treated with α -cypermethrin (Fastac EC/Dominex EC). Controls were treated with Agral only. The data were analysed using probit analysis²⁶ revised for

Table 1. Composition of microencapsulated formulations^a

Microencapsulation	PBO (g kg ⁻¹)	α -Cypermethrin (g kg ⁻¹)	PBO: α -cyp ratio
CdC51	230	190	1:1
CdC09	430	220	2:1
CdC45	190	70	2.5:1
CdC43	220	50	4.5:1
CdC27	310	50	6:1
CdC46	220	30	7.5:1
CdC29	230	30	7.5:1
CdC35	225	25	9:1

^a Each also contained 20–30 g kg⁻¹ of the surfactant, Solitem.

microconfiguration in the statistical programme PC PoloPlus (LeOra Software, Berkeley, CA) to generate LD₅₀, LD₉₅, LD₉₉ (including fiducial limits) and slope values. Control mortality was corrected using Abbott's formula.²⁷ The results of the probit analyses were used to generate resistance factors (RFs), calculated as the resistant LC₅₀ divided by the susceptible LC₅₀ (see Tables 2–6).

2.4 Field trial

Formulations of α -cypermethrin, PBO, PBO mixed with α -cypermethrin, CdC09 and a water control were sprayed onto Bollgard II cotton plants (Sicot 289b, 12–14 plants m⁻²) in the field at Emerald, Queensland, Australia, using a hand sprayer calibrated for an output of 400 mL 20 m⁻², with a walking speed of 0.5 m s⁻¹. The conditions at the time of spraying were: wind speed below 10 km h⁻¹, maximum temperature 25 °C, relative humidity 40% and approximately 80% cloud cover.

There were five plots, each 2 m (two rows of cotton plants) \times 10 m, with 1 m (one row) \times 10 m as a buffer between the plots. Treatments were allocated in a randomised block design: plot 1, water control; plot 2, PBO 80EC; plot 3, α -cypermethrin (Dominex EC); plot 4, tank mix of PBO 80EC and α -cypermethrin (Dominex EC); plot 5, CdC09. In all plots the α -cypermethrin:PBO ratio was 1:2; α -cypermethrin was applied at the registered rate for cotton in Australia against *H. armigera* (50 g AI ha⁻¹).

Adult *B. tabaci* pressure was high and uniformly distributed throughout the plots.

Numbers of adult whitefly were counted on the leaves of each terminal to the fifth node prior to treatment and 24 h after treatment. The mean

numbers of adult *B. tabaci*/terminal/plot and 95% confidence intervals were calculated.

3 RESULTS AND DISCUSSION

The results of probit analyses of leaf dip bioassay data for the two aphid species *M. persicae* and *A. gossypii* are given in Table 2. For *M. persicae* the LC₅₀ for α -cypermethrin on the resistant clone 794JZ was 3460 mg L⁻¹ compared with 5.4 mg L⁻¹ for the susceptible clone, giving an RF value of 640. However, when PBO was added to the α -cypermethrin, the RF was reduced to 12 and reduced still further when the PBO was either applied before the insecticide (RF = 2) or when the two were applied as a microencapsulation, allowing release of the PBO before the α -cypermethrin (RF = 3.7). A similar trend was seen for *A. gossypii* where, although the RF to α -cypermethrin for the resistant clone was lower at 12, this was still reduced by pretreatment with PBO (RF = 0.5) or microencapsulation (RF = 0.6). Thus, for both aphid species, the microencapsulated formulation was equally potent to the pretreatment, and they both led to a significant reduction in the resistance factor, resulting in the α -cypermethrin being as effective as against the susceptible clone.

A similar effect was seen in *H. armigera*, where a resistant strain (Australia HAC) with an RF of 86 was reduced to 50 when PBO was applied with α -cypermethrin as a mixture, and to complete sensitivity when the PBO was microencapsulated with the pyrethroid (Table 3).

For *B. tabaci* a resistant strain, PIRGOS, with B-type esterase, had a very high RF of 6830 when treated with α -cypermethrin (Table 4), but this was reduced to 137 when PBO was mixed with the insecticide,

Table 2. Results of probit analyses on leaf dip bioassay data for two aphid species *Myzus persicae* and *Aphis gossypii*^a

Treatment	Species/strain	N	LC ₅₀ (mg L ⁻¹)	95% FL	Slope (\pm SE)	df	χ^2	RF
α -Cypermethrin	<i>M. persicae</i>							
	US1L (S)	260	5.4	2.2–12.5	0.44(\pm 0.07)	24	13.5	1
	794JZ (R)	270	3460.0	340–290 000	0.24(\pm 0.06)	25	19.9	640
	<i>A. gossypii</i>							
	171B (S)	260	5.2	3.2–8.4	0.91(\pm 0.1)	24	10.8	1
	1196 (R)	260	62.9	27.1–199	0.47(\pm 0.07)	24	10.8	12
0.5 g L ⁻¹ PBO + α -cypermethrin mix	<i>M. persicae</i>							
	794JZ (R)	270	62.3	24.1–235	0.44(\pm 0.07)	25	27.1	11.6
	<i>A. gossypii</i>							
	1196 (R)	260	5.6	3.0–10.3	0.65(\pm 0.08)	24	6.7	1.1
0.5 g L ⁻¹ PBO treatment 5 h before α -cypermethrin	<i>M. persicae</i>							
	794JZ (R)	302	10.9	7.3–16.2	1.07(\pm 0.01)	25	9.6	2
	<i>A. gossypii</i>							
	1196 (R)	260	2.4	1.6–3.7	1.16(\pm 0.12)	24	9.9	0.5
CdC35	<i>M. persicae</i>							
	794JZ (R)	260	19.7	10.5–19.9	0.86(\pm 0.09)	24	35.0	3.7
	<i>A. gossypii</i>							
	1196 (R)	260	3.0	2.0–4.4	1.24(\pm 0.13)	24	17.6	0.6

^a N = number of insects tested; LC₅₀ = pyrethroid concentration needed to kill 50%; FL = fiducial limits; SE = standard error; df = degrees of freedom; χ^2 = chi-square value; RF = resistance factor; S = susceptible strain; R = resistant strain.

Table 3. Results of probit analyses on leaf dip bioassay data for first-instar *Helicoverpa armigera* (notation as in Table 2)

Treatment	Strain	N	LC ₅₀ (mg L ⁻¹)	95% FL	Slope (±SE)	df	χ ²	RF
α-Cypermethrin	Susceptible	180	5.8	5.5–6.2	3.0(±0.4)	5	1.4	1
	Australian HAC (R)	180	500	230–1100	1.1(±0.2)	5	5.2	86
0.5 g L ⁻¹ PBO + α-cypermethrin mix	Australian HAC (R)	180	290	150–550	1.5(±0.2)	5	6.6	50
CdC09	Australian HAC (R)	180	3.9	2.6–5.6	2.3(±0.2)	5	0.2	0.6

Table 4. Results of probit analyses on leaf dip bioassay data for *Bemisia tabaci* (notation as in Table 2)

Treatment	Strain	N	LC ₅₀ (mg L ⁻¹)	95% FL	Slope (±SE)	df	χ ²	RF
α-Cypermethrin	SUD-S (S)	898	2.8	2.4–3.3	2.3(±0.2)	24	11.8	1
	PIRGOS (B-type R)	919	19100	4120–740000	1.0(±0.25)	24	29.4	6834
	CHLORAKA (Q-type R)	910	1540	957–3360	1.4(±0.22)	25	4.6	551
0.5 g L ⁻¹ PBO + α-cypermethrin mix	PIRGOS	518	384	290–547	2.1(±0.23)	24	26.8	137
	CHLORAKA	682	1210	749–2400	0.86(±0.1)	25	1.9	433
0.5 g L ⁻¹ PBO treatment 5 h before α-cypermethrin	PIRGOS	544	1.6	1.3–1.9	2.3(±1.0)	24	10.6	0.6
	CHLORAKA	919	8.8	7.7–10.0	3.7(±0.3)	25	6.6	3.1
CdC35	PIRGOS	484	3.6	2.6–4.9	2.3(±0.21)	24	43.8	1.3
	CHLORAKA	508	12.1	8.9–16.7	2.0(±0.21)	13	16.1	4.3

Table 5. Results of probit analyses on leaf dip bioassay data for *Bemisia tabaci* strains DARWIN (susceptible) and EMERALD (B-type R) (notation as in Table 2)

Treatment	Strain	N	LC ₅₀ (mg L ⁻¹)	95% FL	Slope (±SE)	df	χ ²	RF
α-Cypermethrin	Darwin, native (S)	360	0.084	0.072–0.099	3.5(±0.3)	5	1.02	1
	Emerald (B-type R)	360	70	40–120	1.1(±0.2)	5	8.9	833
0.5 g L ⁻¹ PBO + α-cypermethrin mix	Emerald	360	12	8–20	0.93(±0.21)	5	5.8	143
	Emerald	360	0.41	0.17–0.95	1.9(±0.2)	5	2.3	4.8

and to 0.6 and 1.3 for the PBO pretreatment and microencapsulation respectively. For another *B. tabaci* strain, CHLORAKA, with Q-type esterase, the RF for α-cypermethrin was 551 and the addition of PBO to the insecticide mix had little effect on the resistance factor (433). However, when PBO was used as a pretreatment, the RF for α-cypermethrin was reduced to 3.1 and for the microencapsulation to 4.3, so clearly temporal synergism with PBO still proved to be very effective. Similarly, EMERALD had an RF of 833, reduced to 143 with a mix of PBO and α-cypermethrin, and with CdC09 (microencapsulation) the RF was reduced further to 4.8 (Table 5).

These results have demonstrated that, in all four insect species, resistance resulting from enhanced metabolic activity can be effectively overcome when PBO inhibits the enzymes prior to the active ingredient being applied (in the PBO pretreatment) or released (in the microencapsulation). In the leaf dip bioassays of *M. persicae*, *A. gossypii* and *B. tabaci* (Tables 2 and 4) the microencapsulated formulation (CdC35) contained PBO and α-cypermethrin at a ratio of 9:1, whereas for the *H. armigera* assay using CdC09 (Table 3) the ratio was 1:2. To see whether this ratio is important in obtaining maximum efficacy of the product, a range of formulations were tested against the PIRGOS strain of *B. tabaci* (Table 6). When the RFs were plotted against the ratio of PBO to

α-cypermethrin (Fig. 1) it was revealed that the RF values were inversely proportional to this ratio. Thus, greater mortality of the resistant whitefly was obtained when higher proportions of PBO were present in the formulation.

Having established that microencapsulated formulations of PBO and α-cypermethrin mimic the pre-spray

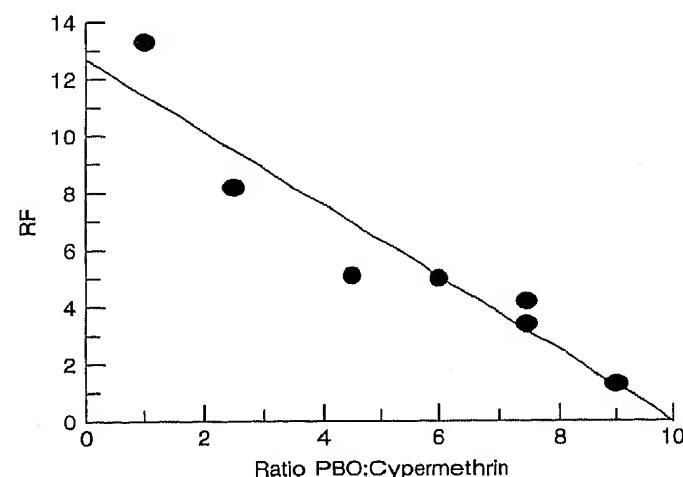
**Figure 1.** Resistance factors for *Bemisia tabaci* strain PIRGOS (B-type R) plotted against the PBO:α-cypermethrin ratio in a microencapsulated formulation (correlation coefficient = 0.944).

Table 6. Results of probit analyses on leaf dip bioassay data for *Bemisia tabaci* strain PIRGOS (B-type R) (notation as in Table 2)

Treatment	PBO: α -cypermethrin ratio	N	LC ₅₀ (mg L ⁻¹)	95% FL	Slope (\pm SE)	df	χ^2	RF
CdC51	1:1	631	37.2	30.4–45.3	2.06(\pm 0.17)	25	3.68	13.3
CdC45	2.5:1	530	22.3	18.9–27.9	2.5(\pm 0.24)	24	15.35	8.2
CdC43	4.5:1	491	14.2	11.5–17.0	2.89(\pm 0.32)	24	9.21	5.1
CdC27	6:1	696	13.9	12.1–15.7	4.4(\pm 0.48)	24	0.2	5
CdC46	7.5:1	518	9.38	7.32–12.1	1.46(\pm 0.11)	24	19.2	3.4
CdC29	7.5:1	520	11.7	9.9–14.1	3.3(\pm 0.35)	24	27.53	4.2
CdC35	9:1	484	3.58	2.64–4.9	2.26(\pm 0.21)	24	43.8	1.3

regime and were very effective at restoring the efficacy of the pyrethroid against insects with esterase-based resistance in leaf dip bioassays, the concept was extended to determine whether similar increases in efficacy could be replicated in field situations. The chosen test system was a field crop of cotton growing in Australia and containing high numbers of Australian B-type *B. tabaci*. The results of the trial are given in Fig. 2. The pre-/post-spray numbers of *B. tabaci* were not statistically different in any treatment, except for the PBO/ α -cypermethrin mixture (in which the mean number of whitefly fell from 9 to 5) and the microencapsulated formulation which gave almost complete control of the insects (12 whitefly to 1).

This study has demonstrated that metabolic-based resistance capable of conferring very high resistance factors can be overcome using temporal synergism. The delay required to allow optimum inhibition of the resistance-associated enzymes can be imparted by bespoke formulations that enable the release of PBO prior to a burst release of insecticide several hours later. Such microencapsulated formulations restored susceptibility to several crop pests, and in at least one, *B. tabaci*, this effect could be repeated in the field. The concept of targeting the defence mechanism(s) of pest species by means of temporal synergism offers the

potential for restoration of insecticides abrogated by metabolic resistance.

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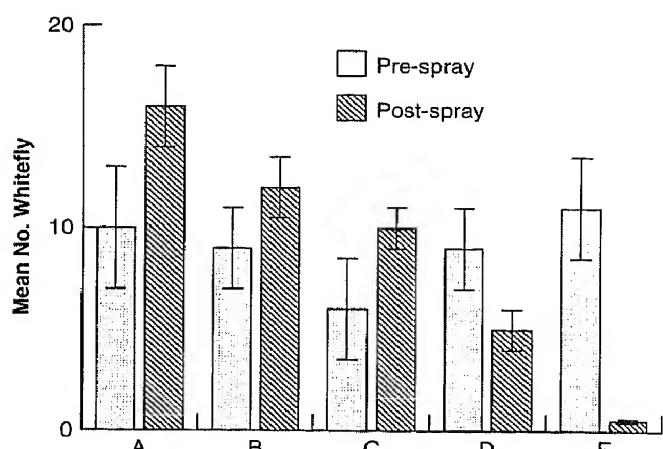


Figure 2. Mean numbers of adult *Bemisia tabaci* present in treatment plots in the field trial (see Section 2.4). Treatments: A = control, water; B = PBO, 500 mg L⁻¹ spray; C = α -cypermethrin, 250 mg L⁻¹ spray; D = PBO 500 mg L⁻¹ and α -cypermethrin 250 mg L⁻¹ spray; E = CdC09, PBO 500 mg L⁻¹ and α -cypermethrin 250 mg L⁻¹ microencapsulated formulation.

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